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DOI: <https://doi.org/10.1007/s00705-008-0262-z>

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ZORA URL: <https://doi.org/10.5167/uzh-30544>

Journal Article

Published Version

Originally published at:

Kwok, T; Helfer, H; Alam, M I; Heinrich, J; Pavlovic, J; Moelling, K (2010). Inhibition of influenza A virus replication by short double-stranded oligodeoxynucleotides. *Archives of Virology*, 154(1):109-14.

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Inhibition of influenza A virus replication by short double-stranded oligodeoxynucleotides

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Received: 12 August 2008 / Accepted: 28 October 2008 / Published online: 26 November 2008
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Abstract Influenza A virus causes prevalent respiratory tract infections in humans. Small interfering RNA (siRNA) and antisense oligonucleotides (asODNs) have been used previously for silencing the RNA genome of influenza virus. Here, we explored the use of partially double-stranded oligodeoxynucleotides (dsODNs) to suppress the production of influenza A virus in cell cultures and animal models. We were able to inhibit influenza A virus replication in cultured human lung cells as well as in the lungs of infected C57BL/6 mice by treatment with dsODN 3-h post-infection. In about 20% of the cases (15/77) the titer was reduced by 10- to 100-fold and in 10% up to 1,000-fold. The antiviral effects of dsODNs were dose-dependent, sequence-dependent and comparable to those of its antisense and siRNA analogues. Thus, dsODNs may be developed as an additional class of nucleic acids for the inhibition of influenza virus replication.

Many RNA viruses and retroviruses, such as influenza A virus, Ebola virus and HIV, are highly infectious agents which cause severe diseases in humans. The RNA genomes of these viruses have been important targets of nucleic-acid-based gene silencing agents such as antisense oligonucleotides and small interfering RNA [1]. We have recently applied a different group of oligonucleotides, referred to as partially double-stranded, hairpin-loop-structured oligodeoxynucleotides, to target HIV-1 RNA

[2–6] as well as the RNA of spleen focus-forming virus in a mouse leukemia model [7]. The antisense strand of the dsODN is complementary to the 3' polypurine tract (PPT) of the retrovirus; it forms a DNA/RNA hybrid with the retroviral RNA, which then activates the retroviral reverse transcriptase/ribonuclease H (RT/RNase H) to cleave at the PPT 5' to the ACU sequence, triggering a premature degradation of the viral RNA and hence abrogation of viral replication [8]. Here, we investigated the ability of dsODNs to induce the degradation of influenza RNA and hence the abrogation of influenza A production in cultured human lung cells and mouse lung.

Influenza A virus, a member of the family *Orthomyxoviridae*, possesses eight RNA genome segments (PB1, PB2, PA, NP, HA, M, NS and NA), which are coated by the nucleoprotein NP [9]. During influenza replication, the virion RNA (vRNA, – strand) is transcribed into mRNA (+ strand) and complementary RNA (cRNA, + strand), which serve as templates for the synthesis of viral proteins and new vRNA, respectively. Influenza virus RNA polymerase plays an essential role in viral replication. Its activities include binding of capped RNA, endonuclease cleavage, and RNA-directed RNA transcription activity. Furthermore, the RNA polymerase of influenza virus is highly conserved among influenza viruses A, B, and C [10]. Thus, influenza virus RNA polymerase is a promising target for inhibition of viral replication. Influenza virus RNA polymerase consists of three subunits, PB1, PB2 and PA. The dsODNs analyzed in this study target the mRNA and cRNA of the PA gene (Fig. 1a). The dsODNs contain three thioated nucleotides at either end and four in the thymidine T4 linker. ODN A, a dsODN that targets the PPT region of the HIV-1 genomic RNA, served as control (Fig. 1a) [2–6]. The vertical bars indicate Watson–Crick bonds, indicating that the ODNs are hairpin-looped structures.

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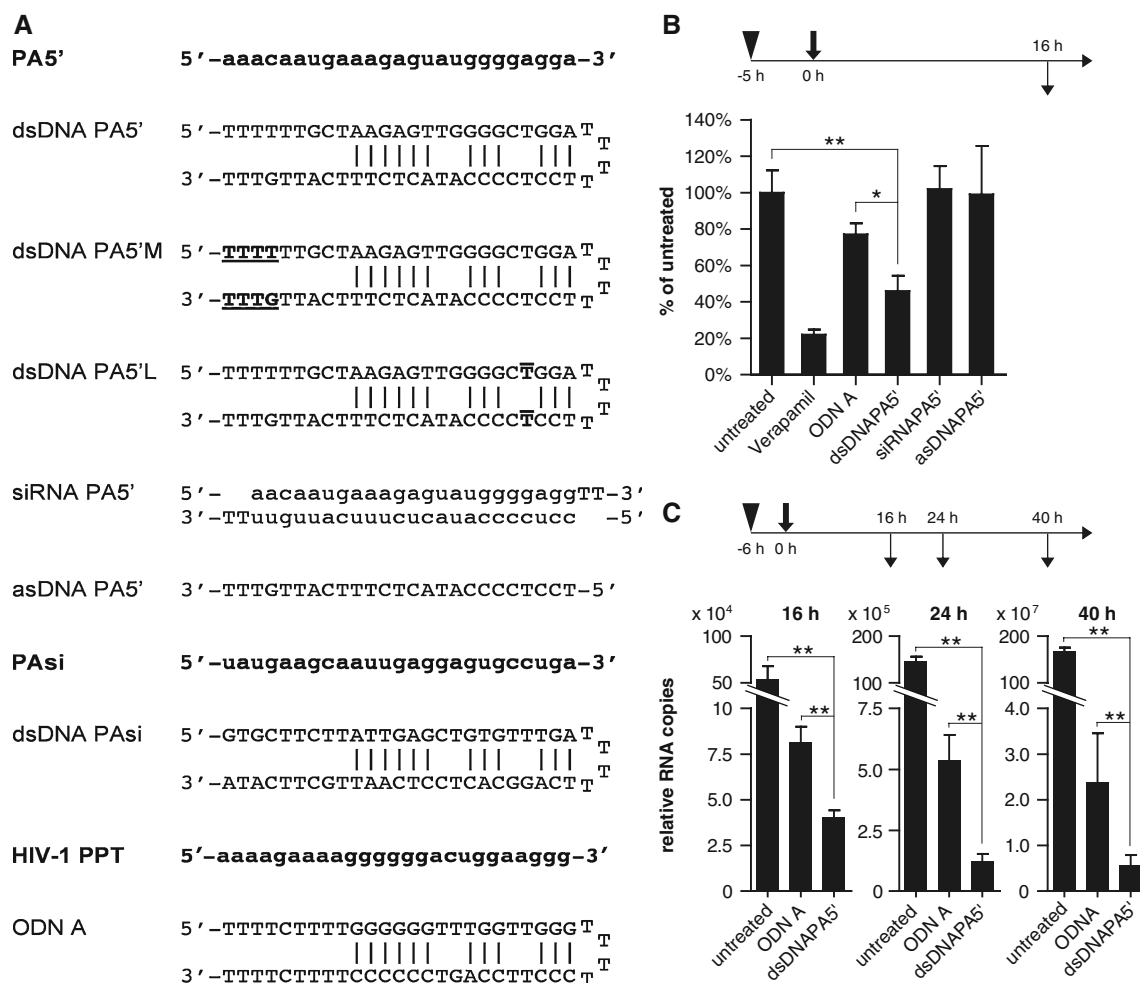


Fig. 1 **a** The oligonucleotides used in this study. The target sequences of the two regions, PA5' and PA5i, of the PA gene in the influenza mRNA and cRNA are shown in bold. PA5i was selected previously for siRNA targeting by PA-2087 [13]. The Watson–Crick bonds are represented by vertical bars. In dsDNA PA5'M, the 2'-O-methylated bases are *underlined*. In dsDNA PA5'L, the locked-nucleic-acid (LNA)-modified bases are *overlined*. ODN A targets HIV-1 genomic RNA and served as a control. Capital letters indicate deoxyribonucleotides, lower-case letters ribonucleotides. All ODNs had three thioate-modified nucleotides at either end and four within the thymidine T4 linker. **b** Short double-stranded DNA (dsDNA PA5') targeting the PA gene specifically inhibited replication of influenza A virus in vitro. The time of transfection of the ODNs is indicated by an arrowhead. Infection is defined as time point 0 h and indicated by a thick arrow on the time line. Thin arrows indicate the times at which the supernatants were collected and the virus titer was determined. The A549 lung cells were grown to 90% confluency and transfected with the various oligonucleotide-Lipofectamine 2000 complexes (80 pmol nucleic acid and 3.3 or 4 μ l Lipofectamine 2000 for DNA or RNA, respectively) 5 h prior to infection. Untreated controls received only medium. The cells were washed twice with phosphate-buffered saline

(PBS) before infection with influenza virus A/PR/8/34 at MOI 0.2. Two-hour post-infection, Verapamil, a previously described inhibitor of influenza virus [12] was added to the medium at a non-toxic concentration (100 μ M). Sixteen hours after infection, supernatants were collected for RNA isolation, as indicated by a thin arrow. Relative amounts of influenza viral RNA were determined by real-time reverse transcriptase polymerase chain reaction (RT-PCR) using primers and probes specific for the NP gene; $n = 6$. Data are expressed as mean \pm SE of the mean (SEM). Statistical significance was determined using the Student's unpaired two-tailed t -test. A probability of $P \leq 0.05$ is considered significant and is indicated with an asterisk. Double asterisks denote $P \leq 0.01$. **c** In vitro kinetics of influenza A replication in cells treated with dsDNA. The sequence of events is summarized by the time line, as described in Fig. 1b. A549 cells were grown to 70% confluency. The cells were transfected with complexes of 8 μ l Lullaby and 80 pmol oligonucleotides. Six hours after transfection, the cells were washed twice with PBS and infected with influenza virus A/PR/8/34 at MOI 0.01. Supernatant was collected at 16-, 24- and 40-h post-infection for the quantitation of influenza RNA; $n = 6$. Data are expressed as mean \pm SEM. Statistical significance was determined as described in Fig. 1b

We first examined the effects of dsDNA PA5' (Fig. 1a) on influenza virus replication in MDCK cells, Vero cells (data not shown) and the human lung cell line A549 [11]. The cells were pre-treated with either dsODN PA5', siRNA PA5', asDNA PA5' or ODN A (Fig. 1a) as negative control

in the presence of Lipofectamine 2000 (Invitrogen). After 5 h, the cells were infected with influenza A/PR/8/34 at a multiplicity of infection (MOI) of 0.2. The calcium channel blocker Verapamil (Biomol), recently shown to exert a strong anti-influenza A virus activity [12], was added to the

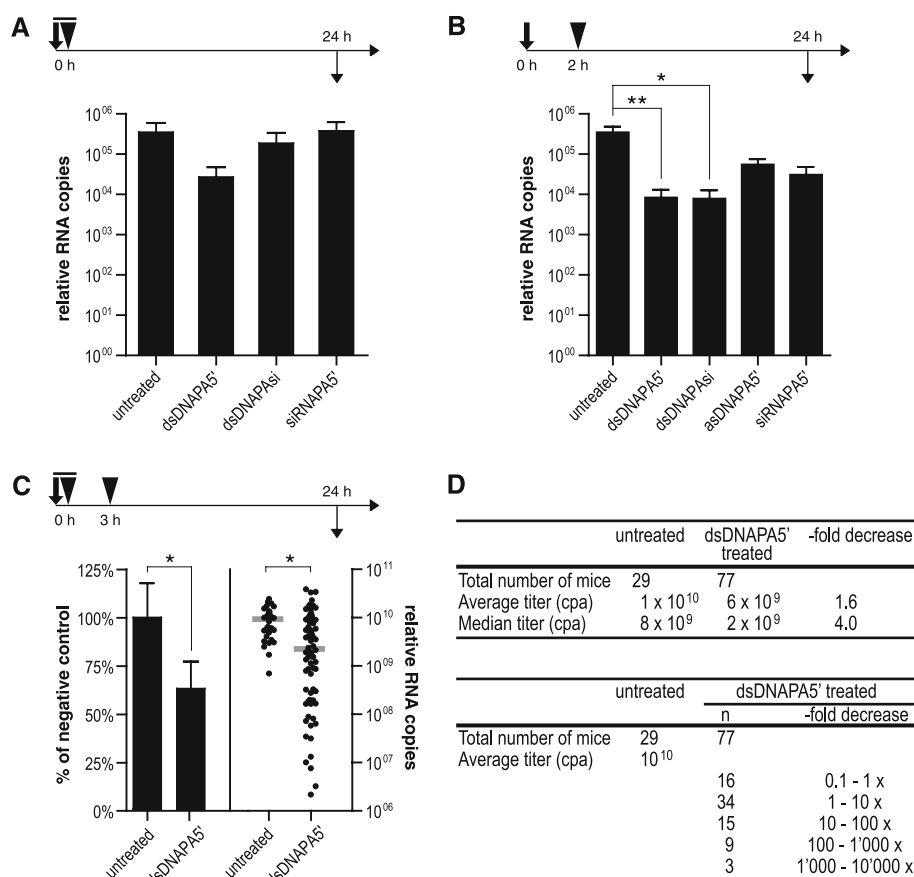


Fig. 2 dsDNAs inhibited the replication of influenza A virus in mouse lungs. The time lines show time of infection at 0 h (*thick arrow*), time points of treatment (*arrowheads*) and lung harvesting for PCR analysis (*thin arrows*). Simultaneous events are *overlined*. **a** Simultaneous therapy. The oligonucleotide-Lipofectamine 2000 complex (2.5 μ l Lipofectamine 2000 and 1 μ g nucleic acid) was mixed with 200 TCID₅₀ of influenza virus A/PR/8/34. The resultant mixture (20 μ l) was applied intranasally. The untreated control consisted of a PBS-virus mixture. Lungs were harvested 24 h after infection for the extraction of total RNA and PCR analysis. Groups of five mice ($n = 5$) were analyzed. Relative amounts of influenza RNA were determined as described in Fig. 1b. **b** Single-dose therapy at 2-h post-infection. A dose of 200 TCID₅₀ of influenza virus A/PR/8/34 in 20 μ l was administered intranasally. Two hours after infection, the oligonucleotide-Lipofectamine 2000 complex (20 μ l) was administered intranasally. Lungs were harvested 24 h after infection for quantitation of influenza RNA; $n = 5$ –10 per group. The data shown are combined from two different experiments. Statistical analysis of the data was performed by determining the variance with the Bonferroni post hoc test applied to logarithmically transformed data using SPSS 16.0 (SPSS Inc. IL). Statistical significance is indicated by a *single asterisk* ($P \leq 0.05$) and a *double asterisk* ($P \leq 0.01$).

medium 2-h post-infection at a non-toxic concentration (100 μ M) and served as positive control. At 16-h post-infection, viral RNA was isolated from the culture supernatant, reverse transcribed into cDNA using random primers and then quantified by real-time polymerase chain reaction (PCR) using primers and probes specific for NP cDNA. Statistical analysis was carried out using either the

c Double-dose therapy. Suppression of influenza virus replication with dsDNA PA5' is shown in comparison to PBS (untreated) control. A mixture (40 μ l) of 300 TCID₅₀ of influenza virus A/PR/8/34 and oligonucleotide-lipofectamine complex was administered intranasally as described in **a**. Three hours after infection, a second dose of oligonucleotide-lipofectamine complex (20 μ l) was administered intranasally. Mouse lungs were harvested 24 h after infection for the quantitation of influenza virus RNA. The data shown are combined from three different experiments. $n = 15$ –40 per group. Viral RNA copies were determined for left and right lungs individually (represented by individual *dots*). Statistical significance is indicated by an asterisk ($P \leq 0.05$) and was determined as described in Fig. 1b. Data are expressed as mean + SEM in bar charts on a linear scale (*left*) and individual dots with median (numerical middle value of a group of data points) on a logarithmic scale (*right*). **d** *Top* Mean and median values were calculated from the titers in copies per assay (cpa). The -fold decrease is the ratio of titers of untreated versus treated mice. **d** *Bottom* Based on the mean value of 10^{10} cpa in the untreated group, mice of the treated group were categorized by viral load. Groups are expressed as -fold reduction. A -fold decrease smaller than 1 indicates an increase

Student's *t*-test or analysis of variance with the Bonferroni post hoc test. Statistical significance is indicated by one asterisk ($P \leq 0.05$) or two asterisks ($P \leq 0.01$). In comparison to the untreated or ODN A-treated controls, pretreatment of A549 cells with dsDNA PA5' resulted in a significant 54% ($P = 0.005$) and 40% ($P = 0.014$) inhibition of virus growth (Fig. 1b). We did not observe any

significant reduction of virus growth by siRNA PA5' or asDNA PA5' (Fig. 1b). To compare the effect under different transfection conditions, Lipofectamine 2000 was replaced with another transfection reagent, Lullaby (Oz Biosciences). A similar inhibition of virus growth was observed with dsDNA PA5' compared to ODN A at 16 h (50%, $P = 0.001$), 24 h (77%, $P = 0.004$), and 40-h post-infection (81%, $P = 0.014$) (Fig. 1c). Although treatment with Lullaby and ODN A led to reduced viral RNA levels compared to untreated samples, the fact that these inhibitory effects were consistently weaker than those observed with dsDNA PA5' (Fig. 1c) suggests that the inhibition by dsDNA PA5' was sequence-dependent. The significant inhibitory effect observed with the ODN A-Lullaby complex suggests the possibility of an off-target effect. To assess toxicity, dsDNA PA5' and asDNA PA5' were tested at tenfold higher amounts (800 pmol) in the absence of any transfection agents. No cytopathic effect was observed. Moreover, application of either compound (80 pmol) to human A549 cells in the presence of a transfection agent did not induce a detectable interferon type I response as tested by immunofluorescence analysis of the interferon-inducible Mx protein (data not shown).

Next, we analyzed the *in vivo* efficacy of dsDNA PA5' in influenza-virus-infected C57BL/6 mice. Intranasal administration of dsDNA PA5' resulted in a 40–98% reduction of influenza virus production in the mouse lungs, depending on the therapeutic regimen used (Fig. 2a–c). Simultaneous administration of dsDNA PA5' and virus resulted in a tenfold reduction of viral RNA in the lungs (Fig. 2a), while a single treatment with dsDNA PA5' 2-h post-infection reduced viral RNA more than 97% ($P = 8.4 \times 10^{-5}$) (Fig. 2b). Interestingly, the double therapy in which the dsDNA PA5'-Lipofectamine 2000 complex was administered both simultaneously with influenza A virus and at 3-h post-infection, did not result in a further increase of the antiviral effect compared to a single treatment at either 0- or 2-h post-infection. Ge and co-workers reported previously that the siRNA PA-2087, which targets the 3' region of the PA mRNA, resulted in a more than 15-fold inhibition of influenza virus A/PR/8/34 and A/WSN/33 replication in Madin-Darby canine kidney (MDCK) cells and a roughly tenfold inhibition in the lungs of C57BL/6 mice [13, 14]. We hence tested the inhibitory effect of dsDNA PA5i, which targets the same region of PA mRNA as PA-2087. dsDNA PA5i was capable of a slight inhibition in the simultaneous treatment (Fig. 2a) and a stronger inhibition of influenza virus replication when administered at 2-h post-infection (Fig. 2b). As evidenced in Fig. 1b and c, treatment with HIV-1-specific ODN A control clearly lead to the reduction of influenza A virus titer by an unknown, target-sequence-independent mechanism. As mentioned previously [7, 15], ODN A can form high-molecular-weight

structures, possibly G-tetrads. However, very recent observations show that dsDNA PA5' exists only in the monomeric form (Heinrich and Moelling, unpublished data). The sequence-independent inhibitory effect of ODN A against influenza A virus may be explained by its oligomeric structures, making it a poor negative control. For this reason we have omitted ODN A treatment as negative control in the animal experiments shown in Figs. 2 and 3.

Using larger numbers of animals per group ($n = 15$ –40), it was confirmed that the inhibitory effect of dsDNA PA5' was significant when compared to an untreated control ($P = 0.043$) (Fig. 2c). The reduction of the average titer was twofold and that of the median fourfold (Fig. 2c, d). A high variability is observed, which may be attributable to the nasal treatment of the mice, which is not easily controlled and required a large number of animals. Moreover, 20% (15/77) of the dsDNA PA5'-treated lungs showed a 10-to-100-fold reduction of viral RNA, and 10% (9/77) of the treated mice exhibited a reduced viral titer in the lungs up to 1,000-fold. A few mice (3/77) even showed a reduction of 1,000- to 10,000-fold (Fig. 2d). None of the ODNs administered to the mice described in Figs. 2 and 3 led to an overt increase of the size of liver or spleen, even when 1 μ g of ODN was used, indicating low toxicity. Our results show that dsDNA PA5' exerted a more effective inhibition when the tissue culture infectious dose 50 (TCID₅₀) was 200 compared to TCID₅₀ of 300 or 400 (equals 280 plaque-forming units), indicating that the efficacy of dsDNA PA5' depended on the input viral dose (Fig. 3a). In addition, dsDNA PA5' inhibited influenza virus production in the lungs in a dose-dependent manner (Fig. 3b). Finally, we investigated whether chemical modifications such as 2'-*O*-methylation (M) or locked-nucleic-acid (LNA) chemistry might be able to further enhance the antiviral effect of dsDNA PA5' (designated dsDNA PA5' M, or dsDNA PA5' L, respectively, shown in Fig. 1a). Analysis of the viral titers in the lung indicated that modification of dsDNA PA5' by 2'-*O*-methylation improved the *in vivo* efficacy of dsDNA PA5' by 75% ($P = 0.019$) (Fig. 3c). In the case of LNA modifications, we did not observe an improvement (data not shown); however, to determine the location and number of modified nucleotides, a more systematic analysis is required.

In summary, we have shown here that a dsDNA designed to target the 5' end of the mRNA of the influenza virus RNA polymerase was able to inhibit influenza virus replication in cell culture as well as in mouse lungs. The observed antiviral effects of dsDNA PA5' were slightly superior to those of asDNA PA5' and siRNA PA5' in the A549 human lung cell line and in lungs of C57BL/6 mice. The anti-influenza virus activity of dsDNA PA5' appears to be primarily sequence-dependent. The higher efficacy of dsODNs compared to single-stranded asDNA or siRNA

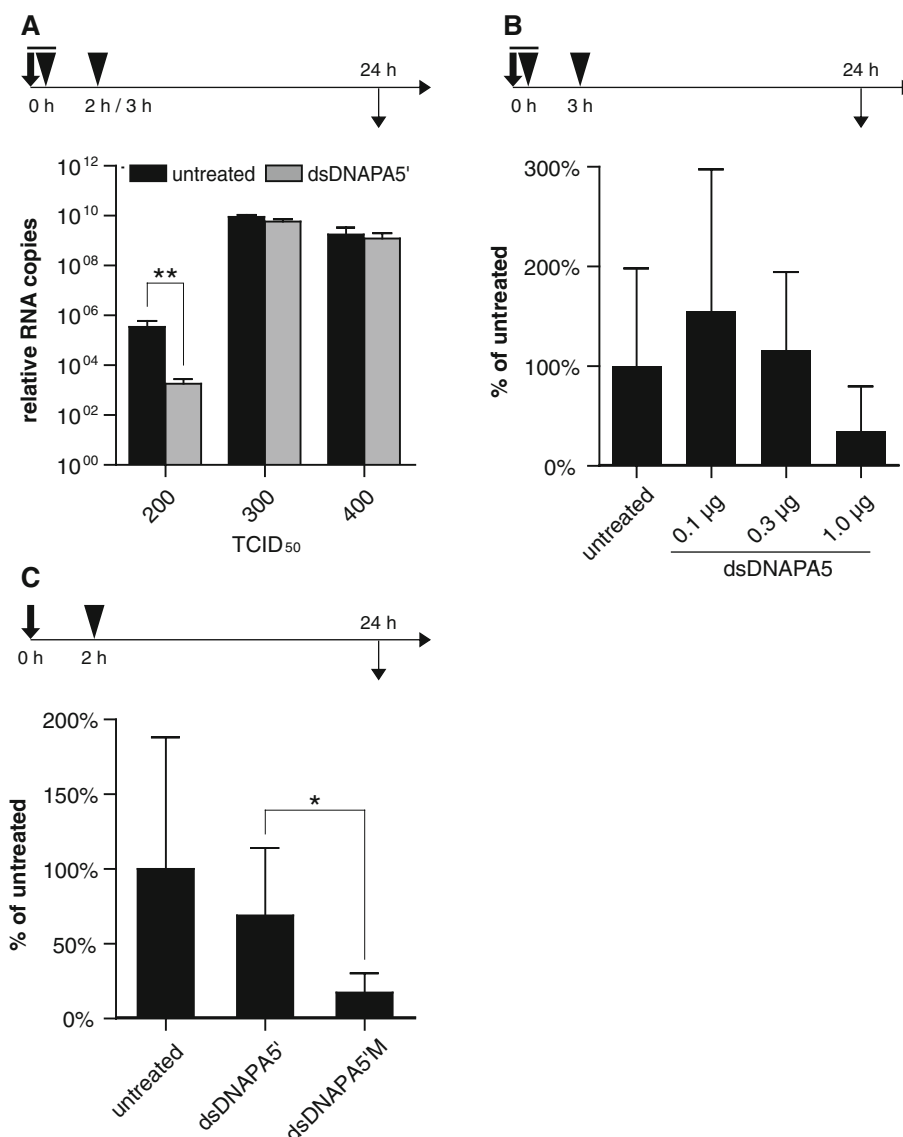


Fig. 3 The effects of viral dose, oligonucleotide dose, and nucleotide sequence in mice. Mice were infected intranasally as described in Fig. 2. ODN (1 μg) was used per treatment unless otherwise stated. The lungs were harvested 24 h after infection for the quantitation of influenza virus RNA. **a** Effects of viral dose. C57BL/6 mice were infected intranasally with 200 (20 μl), 300 (40 μl) or 400 (40 μl) TCID₅₀ of influenza virus A/PR/8/34 and were treated at 0 h and at 2- or 3-h post-infection with dsDNA PA5' or PBS (untreated control) as shown in the time line; $n = 5-15$ per group. Statistical significance is indicated by a double asterisk ($P \leq 0.01$) and was determined as described in Fig. 2b. **b** Dose dependence of dsDNA PA5'. C57BL/6 mice were infected intranasally with 200 or 400 TCID₅₀ of influenza

virus A/PR/8/34 and were treated twice with 0.1, 0.3 or 1 μg (per treatment) of dsDNA PA5' or PBS (untreated control) as shown in the time line. The data shown are combined from two different experiments. $n = 5-10$ per group. **c** 2'-O-methyl modification (M) of dsDNA PA5' (see Fig. 1a). C57BL/6 mice were infected intranasally with 400 TCID₅₀ (40 μl) of influenza virus A/PR/8/34 and were treated at 2-h post-infection with 1 μg of dsDNA PA5' or dsDNA PA5'M complexed with Lipofectamine 2000. The lungs were harvested 24 h after infection for the quantitation of influenza virus RNA; $n = 5$ per group. Statistical significance is indicated by an asterisk ($P \leq 0.05$) and was determined as described in b. The data are shown as mean + SEM

may be due to more favorable in vivo pharmacokinetics and protection against nucleases by the hairpin-loop structure. Ge and co-workers reported previously that intranasal administration of 60 μg of siRNA PA-2087, which targets the 3' region of the PA mRNA, resulted in an approximately tenfold inhibition in the lungs of C57BL/6 mice [13, 14]. In this study, dsDNA PA5i, the dsDNA variant of

PA-2087, exhibited a similar potency at a dose as low as 1 μg per animal. Interestingly, when administered simultaneously with infection, the efficacy of dsDNA PA5i was lower than that of dsDNA PA5', suggesting that the antiviral effects of dsDNA, like those of siRNA or asODNs, could be determined by the structure and accessibility of the target mRNA. We noticed a faster response of the mice

to dsODN than to siRNA by several hours, which may be explained by the different mechanisms. The dsODN does not require an unwinding step in a RISC particle, and the formation of an RNA/DNA hybrid is thermodynamically more favorable than double-stranded RNA.

In the case of an influenza virus infection it is essential to immediately inhibit viral replication. We demonstrate here that application of dsODN, even 3-h post-infection, reduces the viral load in the lung. The high variability of intranasal infection may even lead to better results in a less variable animal model.

With the use of C57BL/6 mice, which are Mx-deficient and hence incapable of an influenza-specific interferon type I response, our study demonstrates that the inhibition of influenza replication by dsDNAs in mouse lungs occurred via an interferon-independent pathway. Moreover, in cell culture, transfection of the dsDNAs used in this study with Lipofectamine 2000 did not induce a type I interferon response (data not shown). Our findings suggest that dsDNAs exert their anti-influenza effects by inhibiting the production of influenza virus RNA during infection. The antisense strand of dsDNA PA5' is complementary to both the influenza mRNA and cRNA. Our previous studies have suggested that dsODN inhibits HIV-1 replication by forming an RNA/DNA hybrid with the retroviral RNA and hence inducing the RNase H activity of RT to degrade the retroviral RNA [4–6]. Given that the influenza virus genome does not encode an RNase H, we propose that dsDNA PA5' induces degradation of influenza viral RNA via different mechanisms, e.g. by activating cellular RNases H for the degradation of the viral mRNA. We cannot exclude other effects such as translational inhibition. Influenza virus undergoes constant genetic changes. Novel therapeutics against an influenza virus pandemic are thus urgently needed. This study is a proof-of-concept which shows that dsDNAs may complement other classes of nucleic-acid based inhibitors.

Acknowledgments We thank Dr. Walter Bossart and Dr. Mauro Imperiali for help setting up the real-time PCR of influenza virus RNA, Dr. Jochen Heinrich for support with statistical analysis and design of the dsDNAs, Firouzeh Farahmand and Eva Mantei for their expert and diligent technical assistance and Silvana Ressegatti for excellent animal care. The influenza system was established and guided by Dr. Jovan Pavlovic. Dr. Terry Kwok initiated and performed most of the mouse studies and wrote the manuscript. Dr. Mohammad Intakhab Alam contributed to cell-culture and animal studies as well as PCR analysis. Dr. Hanspeter Helfer performed cell-culture studies, PCR analysis, statistical analysis, editing of the manuscript, and preparation of the figures. Prof. Dr. Karin Moelling conceived the approach with dsDNA and accompanied the experiments throughout the studies.

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